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KLAUS J. BACH & ASSOCIATES
4407 TWIN OAKS LANE
MURRYSVILLE PA 15668 USA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re: Patent Application to be filed under 35 U.S.C. par. 120 as a Continuation-In-Part Application of International Patent Application No. PCT/DE96/02485 filed on 18/12/96 and claiming priority of German Patent Application No. 195 48 222.0 filed on 22/12/95.

Docket No.: FJ 122

Inventors: Marina Vrljić, Lothar Eggeling, Hermann Sahm

Title: PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS BY BOOSTED ACTIVITY OF EXPORT CARRIERS

jc523 U.S. PTO
09/105117
06/17/98

Honorable Commissioner of Patents and Trademarks
Washington D.C. 20231
BOX PATENT APPLICATION

June 13, 1998

TRANSMITTAL LETTER FOR NEW PATENT APPLICATION UNDER 35 U.S.C.-119

Sir:

Please find enclosed herewith the following documents relating to the above-identified case:
Priority of German application 195 48 222.0 is claimed

1. Specification including 25 pages with 48 claims, 4 drawings
2. Declaration and Power of Attorney
3. Assignment and Recordation Form Cover Sheet
4. A return postcard for acknowledgement of receipt
5. Certified copy of basic German application to follow

The fees involved are as follows:

Basic fee	Assignment fee	Two Excess Independent claims	Multiple Dependent Claims	Excess Claims Over 20	Total fee
790.--	40.--	44.--	270.--	616.--	1760.--

A check covering the total fees as applicable to the present application is attached.

Respectfully submitted,

K. Bach

Klaus J. Bach
Reg. No. 26,832
Tel.: 412-327-0664

09/105,117

PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS
BY BOOSTED ACTIVITY OF EXPORT CARRIERS
BACKGROUND OF THE INVENTION

The invention relates to a process for the microbial production of amino acids, to export genes, to regulator genes, to vectors, to transformed cells, to membrane proteins, and to uses.

Amino acids are of high economical interest and there are many applications for the amino acids: for example, L-lysine as well as L-threonine and L-tryptophan are needed as feed additives, L-glutamate as seasoning additive, L-isoleucine, and L-tryosine in the pharmaceutical industry, L-arginine and L-isoleucine as medicine or L-glutamate and L-phenylalanine as a starting substance for the synthesis of fine chemicals.

A preferred method for the manufacture of these different amino acids is the biotechnological manufacture by means of microorganisms; since, in this way, the biologically effective and optically active form of the respective amino acid is directly obtained and simple and inexpensive raw materials can be used. As microorganisms, for example, *Carynebacterium glutamicum* and its relatives *ssp. flavum* and *ssp lactofermentum* (Liebl et al; Int. J-System Bacteriol (1991) 41:255-260) as well as *Escherichia coli* and related bacteria can be used.

However, these bacteria produce the amino acids only in the amounts needed for their growth such that no excess amino acids are

No sequence listing submitted on diskette.

Sample of submitted file contents. Per 1.824 of Sequence Rules, the Sequence Listing needs to be submitted as a single file. No other file data should be in the file.

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20 However, these bacteria produce the amino acids only in the amounts needed for their growth such that no excess amino acids are generated and are available. The reason for this is that in the cell the biosynthesis of the amino acids is controlled in various ways. As a result, different methods of increasing the
25 formation of products by overcoming the control mechanisms are already known. In these processes, for example, amino acid analogs are utilized to render the control of the biosynthesis ineffec-

5 tive. A method is described, for example, wherein Coarynebacterium strains are used which are resistant to L-tyrosine and L-phenylalanine analogs (JP 19037/1976 and 39517/1978). Also methods have been described in which bacteria resistant to L-lysine and also to L-threonine analogs are used in order to overcome the control mechanisms (EP 0 205 849 B1, UK patent application GB 2 152 509 A).

10 Furthermore, microorganisms constructed by recombinant DNA techniques are known wherein the control of the biosynthesis has also been eliminated by cloning and expressing the genes which code for the key enzymes which cannot be feed-back inhibited any more. For example, a recombinant L-lysine producing bacterium with plasmid-coded feedback-resistant aspartate kinase is known (EP 0381527). Also, a recombinant L-phenylalanine producing bacterium with feedback resistant prephenate dehydrogenase has been described (JP 124375/1986; EP 0 488 424). In addition, increased amino acid yields have been obtained by overexpression of genes which do not code for feedback-sensitive enzymes of the amino acids synthesis. For example, the lysine formation is improved by increased synthesis of the dihydrodipicolinate synthase (EP 0 197 335). Also, the threonine formation is improved by increased synthesis of threonine dehydratase (EP 0 436 886 A1).

20 Further experiments for increasing the amino acid production aim at an improved generation of the cellular primary metabolites of the central metabolism. In this connection, it is known that the overexpression of the transketolase achieved by recombinant techniques improve the product generation of L-tryptophan, L-tyrosine or L-phenalanine (EP 0 600 463 A2). Furthermore, the reduction of the phosphoenol pyruvate carboxylase activity in Carynebacterium provides for an improvement in the generation of aromatic amino acids (EP 0 331 145).

30 All these attempts to increase the productivity have the aim to overcome the limitation of the cytosolic synthesis of the amino

acids. However, as a further limitation basically also the export of the amino acids formed in the interior of a cell into the culture medium should be taken into consideration. As a result, it has been tried to improve this export and, consequently, the efficiency of the amino acid production. For example, the cell permeability of the *Carynebacterium* has been increased by biotin deficiency, detergence or penicillin treatment. However, these treatments were effective exclusively in the production of glutamate, whereas the synthesis of other amino acids could not be improved in this manner. Also, bacteria strains have been developed in which the activity of the secretion system is increased by chemical or physical mutations. In this way, for example, a *Corynebacterium glutamicum* strain has been obtained which has an improved secretion activity and is therefore especially suitable for the L-Lysine production. (DE 02 03 320).

Altogether, the attempts to increase the secretion of amino acids formed within the cell have all in common that an increase efflux of amino acids on the basis of the selected non-directed and non-specific methods could be achieved only accidentally.

Solely in the German patent application No. 195 23 279.8-41, a process is described which provides for a well-defined increase of the secretion of amino acids formed internally in a cell by increasing the expression of genes coding for the import of amino acids. The understanding on which this process was based, that is, the cell utilizes import proteins for the export of amino acids as well as the fact that by nature microorganisms do not generate and release excess amino acids lets one assume that export genes or proteins specific for the amino acid transport do not exist, but that the amino acids are excreted by way of other export systems.

The export systems known so far export poisonous metal ions, toxic antibiotica and higher molecular toxins. These export systems are relatively complex in their structure. Generally, mem-

brane proteins of the cytoplasmic membrane are involved which how-
ever cause only a partial reaction of the export so that presuma-
bly additional extra cytoplasmic support proteins are needed for
the transport (Dink, T. et al., A family of large molecules across
5 the outer membranes of gram-negative bacteria., J. Bacteriol.
1994, 176: 3825-3831). Furthermore, it is known that, with the
sec-dependent export system for extra-cellular proteins, at least
six different protein components are essential for the export.
This state-of-the-art suggests that also the systems, which are
10 responsible for the export of amino acids, but which are not known
so far comprise several protein components or respectively, sev-
eral genes are responsible for the export of amino acids. A hint
in this direction could be the various mutants which are defective
in the lysine export as described by Vrylic et al., (J. Bacteriol
15 (1995) 177:4021-4027).

SUMMARY OF THE INVENTION

It has now been found surprisingly that only a single spe-
cific gene is responsible for the export of amino acids so that,
in accordance with the invention, for the first time a method for
20 the microbial manufacture of amino acids is provided wherein
clearly the export gene expression and/or the export carrier ac-
tivity of a microorganism producing amino acids is increased. The
increased export expression or respectively, activity of the ex-
port carrier resulting from this process leads to an increased se-
cretion rate so that the export of the respective amino acid is
25 increased. The microorganisms so modified also accumulate an in-
creased part of the respective amino acid in the culture medium.

For an increase in the export carrier activity especially the
endogenic activity of an amino acid producing microorganism is in-
30 creased. An increase of the enzyme activity can be obtained for
example by an increased substrate consumption achieved by changing
the catalytic center or by eliminating the effects of enzyme in-
hibitors. An increased enzyme activity can also be caused by an

increased enzyme synthesis for example by gene amplification or by eliminating factors which inhibit the enzyme biosynthesis. The endogene export activity is increased preferably by mutation of the endogenic export gene. Such mutations can be generated either
5 in an uncontrolled manner in accordance with classic methods as for example by UV irradiation or by mutation causing chemicals or in a controlled manner by gene-technological methods such as deletion(s) insertion(s) and/or nucleotide exchange(s).

The export gene expression is increased by increasing the
10 number of gene copies and/or by increasing regulatory factors which positively affect the export gene expression. For example, a strengthening of regulatory elements takes place preferably on the transcription level by increasing particularly the transcription signals. This can be accomplished for example in that, by
15 changing the promoter sequence arranged before the structure gene, the effectiveness of the promoter is increased or by completely replacing the promoter by more effective promoters. An amplification of the transcription can also be achieved by accordingly influencing a regulator gene assigned to the export gene as will be
20 explained further below. On the other hand, an amplification of the translation is also possible, for example, by improving the stability of the m-RNA.

To increase the number of gene copies the export gene is installed in a gene construct or, respectively, in a vector, preferably, a vector with a small number of copies. The gene construct includes regulatory gene sequences, which are specifically
25 assigned to the export gene, preferably such sequences which reinforce the gene expression. The regulatory gene sequences comprise a nucleotide sequence which codes for the amino acid sequence given in table 1 or the allele variations thereof or respectively,
30 a nucleotide sequence 954 to 82 according to table 2 or a DNA sequence which is effective essentially in the same manner.

Allele variations or, respectively, equally effective DNA

sequences comprise particularly functional derivatives which can be obtained by deletion(s) insertion(s) and/or substitution(s) of nucleotides of corresponding sequences, wherein however the regulator protein activity or function is retained or even increased.

5 In this way, the effectiveness of the interaction of the regulatory protein to the DNA of the export gene to be regulated can be influenced by mutating the regulatory gene sequence such that the transcription is strengthened and, consequently, the gene expression is increased. In addition, also so-called enhancers may be

10 assigned to the export gene as regulatory sequences whereby, via an improved correlation between RNA polymerase and DNA, also the export gene expression is increased.

For the insertion of the export gene into a gene construct, the gene is preferably isolated from a microorganism strain of the

15 type *Corynebacterium* and, with the gene construct including the export gene, a microorganism strain, especially *Corynebacterium*, producing the respective amino acid is transformed. The isolation and transformation of the respective transport gene occurs according to the usual methods. If a transport gene is isolated and

20 cloned from *Corynebacterium* then for example, the method of homologous complementation of an export defective mutant is suitable (J.Bacteriol. (1995)177: 4021-4027). If a direct cloning of the structure gene is not possible vector sequences may first be inserted into the transport gene whereupon it is isolated by way of

25 "plasmid rescue" in the form of inactive fragments. For the process according to the invention genes from the *C. glutamicum* ATCC 13032 or *C. glutamicum* ssp. *flavum* 14067 or also, *C. glutamicum* ssp. *lacto fermentum* ATCC 13869 are particularly suitable. The isolation of the genes and their in-vitro recombination with known

30 vectors (Appl. Env. Microbial (1989)55: 684-688; Gene 102(1991)93-98) is followed by the transformation into the amino acid producing strains by electroporation (Liebl et al. (1989)FEMS Microbiol Lett. 65; 299-304) or conjugation (Schäfer et al. (1990) J. Bacte-

riol. 172:1663-1666). For the transfer, preferably vectors with low numbers of copies are used. As host cells, preferably such amino acid producers are used which are deregulated in the synthesis of the respective amino acids and/or which have an increased availability of central metabolism metabolites.

After isolation, export genes with nucleotide sequences can be obtained which code for the amino acid sequences given in table 3 or for their allele variations or, respectively, which include the nucleotide sequence of 1016 to 1725 according to table 2 or a DNA sequence which is effective essentially in the same way. Also here, allele variations or equally effective DNA sequences include particularly functional derivatives in the sense indicated above for the regulatory sequences. These export genes are preferably used in the process according to the invention.

One or several DNA sequences can be connected to the export gene with or without attached promoter or respectively, with or without associated regulator gene, so that the gene is included in a gene structure.

By cloning of export genes, plasmids or, respectively, vectors can be obtained which contain the export gene and which, as already mentioned, are suitable for the transformation of an amino acid producer. The cells obtained by transformation which are mainly transformed cells from *Corynebacterium*, contain the gene in reproducible form, that is, with additional copies on the chromosome wherein the gene copies are integrated at any point of the genome by homologous recombination and/or on a plasmid or respectively, vector.

A multitude of sequences is known which code for membrane proteins of unknown function. By providing in accordance with the invention export genes such as the export gene with the nucleotide sequence of nucleotide 10165 to 1725 in accordance with table 2 or respectively, the corresponding export proteins for example that with the amino acid sequence according to table 1, it is now pos-

sible to identify by sequence comparison membrane proteins, whose function is the transport of amino acids. The export gene identified in this way can subsequently be used to improve the amino acid production in accordance with the process of the invention.

5 The membrane proteins known from the state-of-the-art generally include 12, some also only 4 transmembrane helices. However, it has now been found surprisingly that the membrane proteins responsible or suitable for the export of amino acids include 6 transmembrane helices (see for example, the amino acid sequence of
10 an export protein listed in the table 3, wherein the 6 transmembrane areas have been highlighted by underlining). Consequently, there is a new class of membrane proteins present which has not yet been described.

BRIEF DESCRIPTION OF DRAWINGS

15 Figure 1 shows the fragments in pMV6-3 and pMV8-5-24 obtained by the cloning which cause the lysine secretion and the subclone pMV2-3 made from pMV6-3, which also causes the lysine secretion and which was sequenced. B₁BamH1; Sm, SmaI; Se, SacI; Sl, Sal I,II, HindII; X, XhoI.

20 Figure 2 shows a comparison of the derivated amino acid sequence of LysE from *C. glutamicum* (above), with a gene product of so far unknown function from *Escherichia coli* (below), which is identified thereby as export carrier.

25 Fig. 3 shows increased lysine export by pMV2-3 with *C. glutamicum* NA8. On top, the control with low excretion and cell-internal backup of lysine up to about 150mM. Below, the high secretion caused by pMV2-3 with cell internally only small backup of about 30mM.

30 Figure 4 shows the increase of the lysine accumulation in *C. glutamicum* by lys E lys G(pMV2-3) (middle curve), and the accumulation caused by lysE(lysE) (upper curve).

Examples:

a) Cloning of an export gene and cloning of a regulator of

Corynebacterium glutamicum.

Chromosomal DNA from *C. glutamicum* R127 (FEMS Microbiol lett. (1989)65:299-304) was isolated as described by Scharzer et al. (Bio/Technology (1990) 9:84-87). The DNA was then split with the restriction enzyme *Sau3A* and separated by saccharose gradient centrifugation as described in Sambrook et al. (Molecular cloning, A laboratory manual (1989) Cold Spring Harbour Laboratory Press). The various fractions were analyzed gel electrophoretically with respect to their size and the fraction with a fragment size of about 6 - 10kb was used for the ligation with the vector pJCl. In addition, the vector pJCl was linearized with *Bam*HI and dephosphorylized. Five ng thereof was ligated with 20ng of the chromosomal 6-10 kb fragments. With the whole ligation preparation, the export defective mutant NA8 (J. Bacteriol. (1995)177:4021-4027) was transformed by electroporation (FEMS Microbiol Lett(1989)65:299 - 304). The transformants were selected for LBHIS(PEMS Microbiol. Lett. (1989)65:299-304) with 15µg kanamycin per ml. These transformants were subjected to extensive plasmid analyses in that 200 of the altogether 4500 clones obtained were individually cultivated and their plasmid content and size was determined. On average, about half of the kanamycin-resistant clones carried a recombinant plasmid with an insert of the average size of 8kb. This provides for a probability of 0.96 for the presence of any gene of *C. glutamicum* in the established gene bank. The 4500 obtained transformants were all individually checked for renewed presence of lysine secretion. For this purpose, the system described by Vrljic for the induction of the L-lysine excretion in *Corynebacterium glutamicum* was utilized (J. Bacteriol (1995) 177:4021-4027). For this purpose, so-called minimal-medium-indicator plates were prepared, which contained per liter 20g (NH₄)₂SO₄, 5g uric acid, 1g KH₂PO₄, 1 g K₂HPO₄, 0.25g MgSO₄·7H₂O, 42 g morpholino propane sulfonic acid, 1ml CaCl₂ (1g/100ml), 750 ml dest., 1 ml Cg trace salts, 1 ml biotin (20µg/100l), pH7, 4% glucose, 1.8mg pro-

tocatechuic acid, 1 mg $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$, 1 mg $\text{MnSO}_4 \times \text{H}_2\text{O}$, 0.1 mg $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.02mg CuSO_4 , 0.002mg $\text{NiCl}_2 \times 6\text{H}_2\text{O}$, 20 g agar-agar, as well as 10^7 cells/ml of the lysine-auxotrophene *C. glutamicum* mutant 49/3. The original 4500 transformants were all individually pinned, by toothpicks onto the indicator plates with, in each case, a check of the original non-excretor NA8 (J.Bacteriol (1995)177:4021-4027) and the original strain R127. At the same time, always 2 plates were inoculated of which only one contained additionally 5mM L-methionine in order to induce the lysine excretion in this way. The indicator plates were incubated at 30°C and examined after 15, 24 and 48 hours. In this way, altogether 29 clones were obtained which showed on the indicator plate provided with methionine a growth court by the indicator strain 49/3. The clones were examined individually and then again as described above, for reestablishment of the growth court. In this way, the two clones NA8 pMV8-5-24 and NA8 pMV6-3 were obtained which had again received the capability to excrete lysine.

From these clones, plasmid preparations were performed as described in Schwarzer et al. (Bio/Technology (1990)9; 84-87). By retransformation in NA8, the plasmid-connected effect of the excretion of L-lysine was confirmed. Both plasmids were subjected to a restriction analysis. Plasmid pMV8-5-24 carries an insert of 8.3 kb, and pMV6-3 one of 9.5 kb. The physical charter of the inserts is shown in Fig. 1.

b) Subcloning of an DNA fragment which reconstitutes the lysine export.

From the insert of the plasmid pMV6-3 individual subclones were prepared utilizing the restriction severing point as determined. In this way, the 3.7 kb *Xho*I-*Sal*I-fragment, the 2.3 kb *Bam*HI-fragment and the 7.2 kb *Bam*HI fragment were ligated with the correspondingly severed and treated vector pJCl (Mol Gen. Genet.(1990)220: 478-480). With the ligation products *C. glutamicum* NA8 was directly transformed, the transformants were

tested for having the lysine excretion properties and the presence of the subclone was confirmed by plasmid preparation and restriction analysis. In this way, the strain with plasmid pMV2-3 (Fig. 1) was obtained as smallest subclone. This fragment resulting in lysine export contains as insert the 2.3kb Bam fragment from pMV6-3.

c) Sequence of the lysine export gene *lys E* and its regulators *lysG*.

The nucleotide sequence of the 2.3kb BamH1 fragment was performed according to the dideoxy-chain termination method of Sanger et al. (Proc. Natl. Acad. Sci USA(1977) 74:5463-5467) and the sequencing reaction with the Auto Read Sequencing kit from Pharmacia (Uppsala, Sweden). The electrophoretic analysis occurs with the automatic laser-fluorescence DNA sequencing apparatus (A.L.F) from Pharmacia-LKB(Piscataway, NJ, USA). The nucleotide sequence obtained was analyzed by the program packet HUSAR (Release 3.0) of the German Cancer Research Center (Heidelberg). The nucleotide sequence and the result of the analysis is presented in Fig. 2. The analysis results in two fully open reading frames (ORF) on the sequenced DNA piece. ORF1 codes for a protein with a length of 236 amino acids, ORF2 codes for a protein with a length of 290 amino acids. The protein derived from ORF1 includes an accumulation of hydrophobic amino acids as they are characteristic for membrane-embedded proteins. The detailed analysis of the distribution of the hydrophobic and hydrophilic amino acids by the programs PHD.HTM (Protein Science(1995)4:521-533) is shown in table 3. It is apparent therefrom that the protein contains six hydrophobic helix areas which extend through the membrane. Consequently, this protein is the searched for exporter of the amino acid L-lysine. The corresponding gene will therefore be designated below as *lysE*. In table 2, it is marked accordingly. ORF2 is transcribed in a direction opposite to ORF1. The sequence analysis shows that ORF2 has a high identity with regulator genes

which are combined as a single family (Ann Rev Microbiol(1993) 597-626). Genes of this family regulate the expression processes of the various genes involved in catabolic or anabolic processes in a positive way. For this reason, ORF2 will below be designated
5 as lysG (Govern=regulating). Because of the coordination and because lysE could be cloned (see a)) and subcloned (see b)) together with lysG, lysG is regulator of lysE and consequently also participates in the lysine export. The gene lysG and the amino acid sequence derived therefrom are also shown in table 2 and, re-
10 spectively, table 1.

d) Identification of an unknown membrane protein from Escherichia coli by sequence comparison.

With the established sequences according to table 3, already existing sequence banks can be searched in order to assign the
15 proteins derived in this way from sequenced areas a certain function. Correspondingly, the amino acid sequence of the lysine exporters consisting of C. glutamicum were compared with derivated protein sequences of all the DNA sequences deposited there utilizing the program packet HUSAR (Release 3.0) of the German Cancer
20 Research Center (Heidelberg). A high homology of 39.3% identical amino acids and 64.9% similar amino acids was found to a single sequence of so far unknown function of E.coli.

The comparison is shown in Fig. 2. The open read frame of E. coli so far not characterized is consequently identified by way of
25 this process as an amino export gene.

e) Increased export of intracellularly accumulated L-lysine.

The strain C. glutamicum NA8 (J. Bacteriol(1995) 177: 4021-4027 was transformed with plasmid pMV2-3 and the L-lysine excretion of the strains was compared. For this purpose, NA8 and
30 NA8pMV2-3 in complex medium were utilized as described in Vrljic et al. (J. Bacteriol (1995)177:4021-40277) and the fermentation medium CGXII (Bacteriol (1993)175:5595-5603 were each separately inoculated. The medium additionally contained 5mM L-methionin in

order to induce the intracellular L-lysine biosynthesis. After cultivation for 24 hours at 30°C on a rotary vibrator at 140 rpm, the cell internal and external L-lysine determinations were performed. For the cell-internal determination silicon oil centrifugations were performed (Methods Enzymology LV(1979) 547-567); the determination of the amino acids occurred by high pressure liquid chromatography (J. Chromat (1983) 266:471-482). These determinations were performed at different times as indicated in Fig. 3. In accordance with the process used the retained cell internal L-lysine is excreted also by pMV2-3 to a greater degree and is accumulated. Accordingly, also the cell internally present L-lysine is greatly reduced. Consequently, the utilization of the newly discovered and described exporter represents a process for greatly improving the L-lysine production.

f) Increased accumulation of L-lysine by lysE or LysEG.

From the subclone pMV2-3 which contains the sequenced 2374bp Bam HI-fragment in pJCI (see figure 1), the lysE carrying 1173 bpPvuII fragment was ligated in pZ1 (Appl. Env. Microbiol(1989)55:684-688) according to the sequence information and in this way, the plasmid plysE was obtained. This plasmid as well as the lysE lysG carrying plasmid pMV2-3 was introduced into C. glutamicum strain d by electroporation wherein the chromosomal areas were deleted. The obtained strains C. glutamicum d pMV2-3, C. glutamicum d plysE, C. glutamicum pJCI were, as described under e) precultivated on a complex medium, then cultivated in production minimal medium CGx11 together with 4% glucose and 5mM l-methionin and samples were taken to determine the accumulated lysine. As apparent from Fig. 4 with lysE lysG an increase of the lysine accumulation with respect to a control sample is achieved. With plysE an extraordinarily increased accumulation of from 4.8 to 13.2 mM L-lysine is achieved with this method.

LEGENDS OF THE TABLES

Table 1: The amino acid sequence of the lysine exporter regulator from *Corynebacterium glutamicum* with the helix-turn-helix
5 motive typical for DNA-binding proteins.

Table 2(three pages): The nucleotide sequence of *C. glutamicum* coding for the lysine exporter and lysine export regulators.

Table 3: the amino acid sequence of the lysine exporter from
10 *Corynebacterium glutamicum* with the identified transmembrane helices TMH1 to TMH6.

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

Table 1

1 MNPIQLDTLL SIIDEGSFEG ASLALSISPS AVSQRVKALE
HHVGRVLVSR

Helix-Turn-Helix-Motiv

51 TQPAKATEAG EVLVQAARKM VLLQAETKAQ LSGRLAEIPL
TIAINADSLS

101 TWFPFVNEV ASWGGATLTL RLEDEAHTLS LLRRGDVLGA
VTREANPVAG

151 CEVVELGTMR HLAIATPSLR DAYMVDGKLD WAAMPVLRFG
PKDVLQDRDL

201 DGRVDGPVGR RRVSI VPSAE GFGEAIRRGL GWGLLPETQA
APMLKAGEVI

251 LLDEIPIDTP MYWQRWRLES RSLARLTDAV VDAAIEGLRP

Table 2

GGTAAACGACTTCCACAATGAGACGGACCGGGTTAAGGACGCCGCTTCTTCACTTTTGG	60
<i>-LysG</i>	120
GGACTTGGAAGTCTTCATTGATTCCGGCGTTAGGGAGCTAACGACGTAGTTGCTGCCG - P R L G E I A A D V V A	180
CAGACACTCAGATCGATCTCTAGATCTAAGGTCCGCGGTAGCAACGGTTATGTAGCCACA D T L R A L S R S E L R W R Q W Y M P T	240
CAGTTACCCATAGAGTAGCTCCTCCTAGTGAAGAGGACGAAATCGTACCCTCGTCGAAC D I P I E D L L I V E G A K L M P A A Q	300
CCAAAGCCCTTCTTCAGGGGTTGGTTCCGGAGCCGCTTAACGGAGTGGTTTGGGAAGGCG T E P L L G W G L G R R I A E G F G E A	360
GCTGCCCTGTACCTATGCGCGGACGCGGGGTGTCCTGGTAGCTGCGCGGGCAGGTCCAG S P V I S V R R R G V P G D V R G D L D	420
TGCCAGAACTTCGTGTAGAAACCCTGGCTTCGCATTCTGCCCGTAGCGTCGGGTTAGATC R D Q L V D K P G F R L V P M A A W D L	480
AAAGGGTAGTTGGTACATCCGTAGGGCGTTACTCCCCCAACGTTACCGGTTACCGCGTA K G D V M Y A D R L S P T A I A L H R M	540
CCAAGGTTCAAGATGATGAAGTGTAGGGCGGTGCCCTAATCGAAGTGCCCAATGGCGAGG T G L E V V E C G A V P N A E R T V A G	600
ATTTTGTAGAGGTGCGGCGTCGTTCCCTATTACACACGCGAAGTAGAAGGTTGCGGTCGCA L V D G R R L L S L T H A E D E L R L T	660
CTCGCAACGAGGTGGGGTTCTTCGATGGAGCAACTTGTGCCCTCCTTTGGTACACCTATC L T A G G W S A V E N F V P P F W T S L	720
GCTTAGACGCAACTACCGCTACCAATTGCCCTAAAGTCGTTCCGCAGGTCTATCAACGCG S D A N I A I T L P I E A L R G S L Q A	780
AAATCAAAGACGAACGTCGTTGTGGTAAAAGGCGCGACGAACGTGTTCTGAAGTGGGCG K T E A Q L L V M K R A A Q V L V E G A	840
AAGCCAACGAAACCGGCCAACCCACGCGCTATGGTTGTGAGCTGGGTGCACTACGAGCTC E T A K A P Q T R S V L V R G V H H E L	900
TCGAAATTGCGGACTGAGTGGCGGCTCCCCCTTTACCTTTCCCGATTCTCCGCGGAAG A K V R Q S V A S P S I S L A L S A G E	

Table 2 (cont.)

RCGS 960
 <---LysG
 CTTCGACGGAAGTAGTTACTA ACTCTCGTTTCACAGGTCAACTTACCCCAAGTA-----5'
 5'---TGCCTTCATCAATGATTGAGAGCAAAGTGTCCAGTTGAATGGGGTTCATGAAGCT
 F S G E D I I S L L T D L Q I P N M

RBS 1020
 ATATTAAACCATGTTAAGAACCAATCATTTTACTTAAAGTACTTCCATAGGTCACGATGGT
 M V
 LysE--->

1080
 GATCATGGAAATCTTCATTACAGGTCTGCTTTTGGGGGCCAGTCTTTTACTGTCCATCGG
 I M E I F I T G L L L G A S L L L S I G

1140
 ACCGCAGAATGTACTGGTGATTAAACAAGGAATTAAGCGCGAAGGACTCATTGCGGTTCT
 P Q N V L V I K Q G I K R E G L I A V L

1200
 TCTCGTGTGTTAATTTCTGACGTCTTTTGTTCATCGCCGGCACCTTGGGCGTTGATCT
 L V C L I S D V F L F I A G T L G V D L

1260
 TTTGTCCAATGCCGCGCCGATCGTGCTCGATATTATGCGCTGGGGTGGCATCGCTTACCT
 L S N A A P I V L D I M R W G G I A Y L

1320
 GTTATGGTTTCCCGTCATGGCAGCGAAAGACGCCATGACAAACAAGGTGGAAGCGCCACA
 L W F A V M A A K D A M T N K V E A P Q

1380
 GATCATTGAAGAAACAGAACCAACCGTGCCCGATGACACGCCTTTGGGCGGTTTCGGCGGT
 I I E E T E P T V P D D T P L G G S A V
 >>>>>>> > < <<<<<<<

1440
 GGCCACTGACACGCGCAACCGGGTGCGGGTGGAGGTGAGCGTCGATAAGCAGCGGGTTTG
 A T D T R N R V R V E V S V D K Q R V W

1500
 GGTAAAGCCCATGTTGATGGCAATCGTGCTGACCTGGTTGAACCCGAATGCGTATTTGGA
 V K P M L M A I V L T W L N P N A Y L D

1560
 CGCGTTTGTGTTTATCGGCGCGCTCGGCGCGCAATACGGCGACACCGGACGGTGGATTTT
 A F V F I G G V G A Q Y G D T G R W I F

1620
 CGCCGCTGGCGCGTTCGCGGCAAGCCTGATCTGGTTCCCGCTGGTGGGTTTCGGCGCAGC
 A A G A F A A S L I W F P L V G F G A A

1680
 AGCATTGTCACGCCCCGCTGTCCAGCCCCAAGGTGTGGCGCTGGATCAACGTCGTCGTGGC
 A L S R P L S S P K V W R W I N V V V A

Table 2 (cont.)

```

. . . . . 1740
      >>>>>> <<<<<<< / orf3
      - N E R T K
5'   C T A C T G G C G T A A C C G G T A G T T T G A C T A C A A C T A C C C A A T C A A A A G C G C C C A A A A
A G T T G T G A T G A C C G C A T T G G C C A T C A A A C T G A T G T T G A T G G G T A G T T T T C G C G G G 5'
V V M T A L A I K L M L M G -
      LysE / >>>>>

. . . . . 1800
C C T T A G C C A C C G G A A G C G G G T T T A C A A C T A C G G C C G C A G C A C C C T T T A G A G T A G C T A G C G
S D T A K A W I N I G A D H S I E D I A
      <<<<<<

. . . . . 1860
G A G G T T G A G C C G C A G T C T T T T G A G G T T C A A C A A C T C A C T T A G T T C C G A C A A C A G G T C G A C
E L E A D S F E L N N L S D L S N D L Q

. . . . . 1920
G A G T T G A C T G C T T C G T G G T T A G T T A C G T G A C C A G T G C C A T A G G C G C G G C A T G A G A G G A A C
E V S S A G I L A S T V T D A G Y E G Q

. . . . . 1980
G A G C G C G T C G T G G G T A C G T T C G C G G T A G A C G C G T T C A C T G A C G G G C G C A A G G A C C C G C T A
E R L V W A L A M Q A L S Q G R E Q A I

. . . . . 2040
C A G T A A C T C G A A C G C C T G G T A T A G T T A T A A C A A G T G C A A G T T G T A C G G G A G T C T G T C C C T
D N L K R V M D I N N V N L M G E S L S

. . . . . 2100
G A A T G G G A C C G A C C G C G C C C T T G G G A G A C C T T A A G G T A G C T C T A T A A C A G G C A C T C G T C
K G Q S A R S G E P I G D L Y K D T L L

. . . . . 2160
C G G G A C G C G T T C A C C A C T C T T T C G T T A C T G C G G T T C T G G T A A C A A C C G T C G A C T G A C G T T
G Q A L P S F A I V G L G N N A A S Q L

. . . . . 2220
G T T C A A G A G T G G C A G T A G C G G G C C A A G G A G G T G G G T T G C T A A T T A C T A C C T T A T C G A A C C
L N E G D D G P E E V W R N I I S Y S P

. . . . . 2280
G A C T A C T T A G T C T T C G C C C G T C G G G A G G A G G C G G T A C T T G A G T C G G C G G A G G C G A C A C T C
Q H I L L P C G E E A M F E A A E A T L

. . . . . 2340
G A G A C C T G G C A T C C T T C T T T A T G G G T G C A T T T C T C G A A A G G T C T G C G T T G T T A C A G T G C
E P G Y S S I G V Y L A K G S A V I D R

. . . . . 2374
      <-orf3/
G T T A C G C A T G T A C C A A A G A A G G T T T C C T C A T A G A
L A Y M T E E L P T D

```

Table 3

1	<u>MVIMEIFITG</u>	<u>LLLGASLLLS</u>	<u>IGPQNVLVIK</u>	<u>QGIKREGLIA</u>
	TMH1			TMH2
51	<u>FLFIAGTLGV</u>	<u>DLLSNAAPIV</u>	<u>LDIMRWGGIA</u>	<u>YLLWFAVMAA</u>
	TMH3			
101	<u>PQIIETEPT</u>	<u>VPDDTPLGGS</u>	<u>AVATDTRNRV</u>	<u>RVEVSVDKQR</u>
	TMH4			TMH5
151	<u>VLTWLNPNAY</u>	<u>LDAFVFIGGV</u>	<u>GAQYGDTGRW</u>	<u>IFAAGAF AAS</u>
	TMH6			
201	<u>AAALSRLSS</u>	<u>PKVWRWINVV</u>	<u>VAVVMTALAI</u>	<u>KLMLMG</u>

CLAIMS

What is claimed is:

1. A process for the microbacterial production of amino acids, wherein the export carrier activity and the export gene-expression of a microorganism producing the respective amino acid is increased.

2. A process according to claim 1, wherein the endogenous export carrier activity of the microorganism is increased.

3. A process according to claim 2, wherein, by mutation of the endogenous export gene, a carrier with higher export activity is generated.

4. A process according to one of the claims 1 to 3, wherein the gene expression of the export carrier is increased by increasing the number of gene copies.

5. A process according to claim 4, wherein, in order to increase the number of copies, the export gene is installed in a gene construct.

6. A process according to claim 5, wherein the export gene is installed in a vector with a low number of copies.

7. A process according to claim 5 or 6, wherein the export gene is installed in a gene construct which includes regulatory gene sequences assigned to the export gene.

8. A process according to claim 7, wherein the regulatory gene sequence includes a nucleotide sequence coding for the amino acid sequence as given in table 1 and the allele variations thereof.

9. A process according to claim 8, wherein the regulatory gene sequence includes one of a nucleotide sequence of nucleotide

954 to 82 according to table 2 and a DNA sequence effective essentially in the same way.

10. A process according one of the claims 5 to 9, wherein a microorganism producing the respective amino acid is transformed with the gene construct including the export gene.

11. A process according to claim 10, wherein a microorganism of the type *Corynebacterium* is transformed with the gene construct including the export gene.

12. A process according to claim 10 or 11, wherein, for the transformation, a microorganism is utilized in which the enzymes which participate in the synthesis of the corresponding amino acids are deregulated.

13. A process according to one of claims 10 to 12, wherein, for the transformation, a microorganism is utilized which contains an increased part of the central metabolism metabolites.

14. A process according to one of claims 4 to 13, wherein the export gene is isolated from a microorganism strain of the type *Corynebacterium*.

15. A process according to one of claims 1 to 14, wherein the export gene sequence is identified by comparison with the sequence of an already known export gene.

16. A process according to claim 15, wherein the amino acid sequence derived from the export gene sequence to be identified is compared with the amino acid sequence given in table 3 or the allele variation thereof.

17. A process according to one of claims 1 to 16, wherein the export gene expression is increased by amplifying the transcription signals.

18. A process according to one of the 1 to 17, wherein as export gene, a gene with a nucleotide sequence coding for the amino acid sequence given in table 3 and the allele variations thereof is utilized.

19. A process according to claim 18, wherein as export gene,

one of the genes with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 and a DNA sequence with essentially the same effects is utilized.

20. A process according to one of the preceding claims 1 to 19 for the manufacture of L-lysine.

21. An export gene coding for an amino acid export carrier.

22. An export gene according to claim 21 with a nucleotide sequence coding for an amino sequence given in table 3 or the allele variation thereof.

23. An export gene according to claim 22 with the nucleotide sequence of nucleotide 1016 to 1725 according to table 2 or a DNA sequence with essentially the same effects.

24. An export gene according to one of claims 21 to 23 with regulatory gene sequences assigned thereto.

25. An export gene according to claim 24, wherein the regulating gene sequence includes a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

26. An export gene according to claim 25, wherein said regulating gene sequence includes a nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

27. A regulator gene suitable for the regulation of an export gene coding for an amino acid and export carrier, including a nucleotide sequence coding for the amino sequence given in table 1 and the allele variations thereof.

28. A regulator gene according to claim 27, with the nucleotide sequence of nucleotide 954 to 82 according to table 2 or a DNA sequence effective essentially in the same way.

29. A gene structure containing an export gene according to one of claims 21 to 24.

30. A gene structure including a regulatory gene sequence according to claim 27 or 28.

31. A vector including an export gene according to one of

claims 21 to 26 or a gene structure according to claim 29.

32. A vector according to claim 31 with a low number of copies.

33. A vector including a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

34. A transformed cell including, in a replicable form, an export gene according to one of the claims 21 to 26 or a gene structure according to claim 29.

35. A transformed cell according to claim 34 including a vector according to claim 31 or 32.

36. A transformed cell according to claim 34 or 35, wherein said cell belongs to the type *Corynebacterium*.

37. A transformed cell according to one of claims 34 to 36, wherein in this cell the enzymes of the amino acid, which participate in the synthesis, are deregulated and wherein the amino acid is removed from the cell by way of the export carrier for which the export gene, which was transferred into the transformed cell, codes.

38. A transformed cell according to one of claims 34 to 37, wherein the cell includes an increased proportion of central metabolism metabolites.

39. A transformed cell including, in replicable form, a regulatory gene sequence according to claim 27 or 28 or a gene structure according to claim 30.

40. A transformed cell according to claim 39, including a vector according to claim 33.

41. A membrane protein with 6 transmembrane helices suitable for the export of amino acids.

42. A membrane protein according to claim 41, including the amino acid sequence given in table 3 wherein table 3 is part of this claim.

43. The use of an export gene for increasing the amino acid production of microorganisms.

ABSTRACT OF THE DISCLOSURE

The invention pertains to a process for the microbial production of amino acids. The process involves boosting the export carrier activity and/or export gene expression of a microorganism which produces the desired amino acid. It was found that a single
5 specific gene is responsible for the export of a given amino acid, and on that basis, a process for the microbial production of amino acids, involving the controlled boosting of the export gene expression and/or export carrier activity of a microorganism was developed, which produces the amino acid. The boosted expression or
10 activity of the export carrier resulting from this process increases the secretion rate and thus increases transport of the desired amino acid.

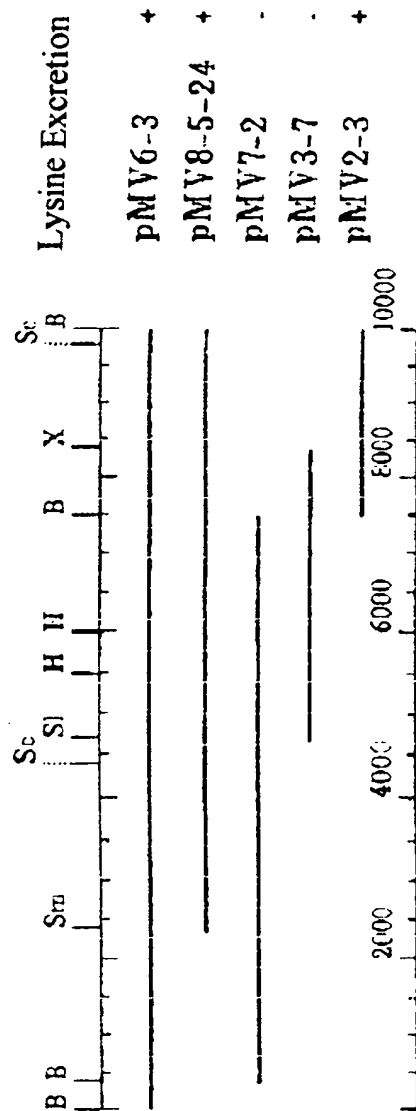


Figure 1

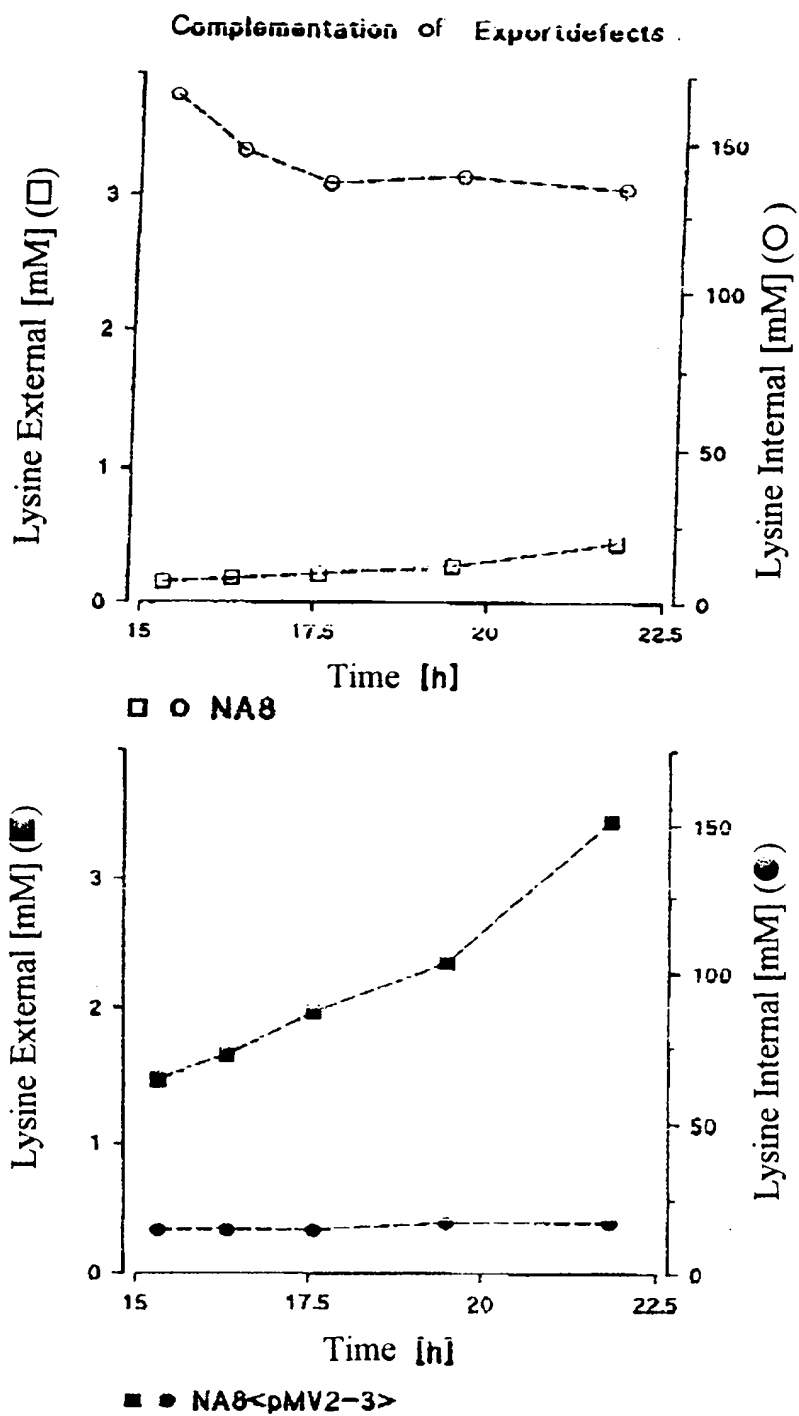


Figure 3

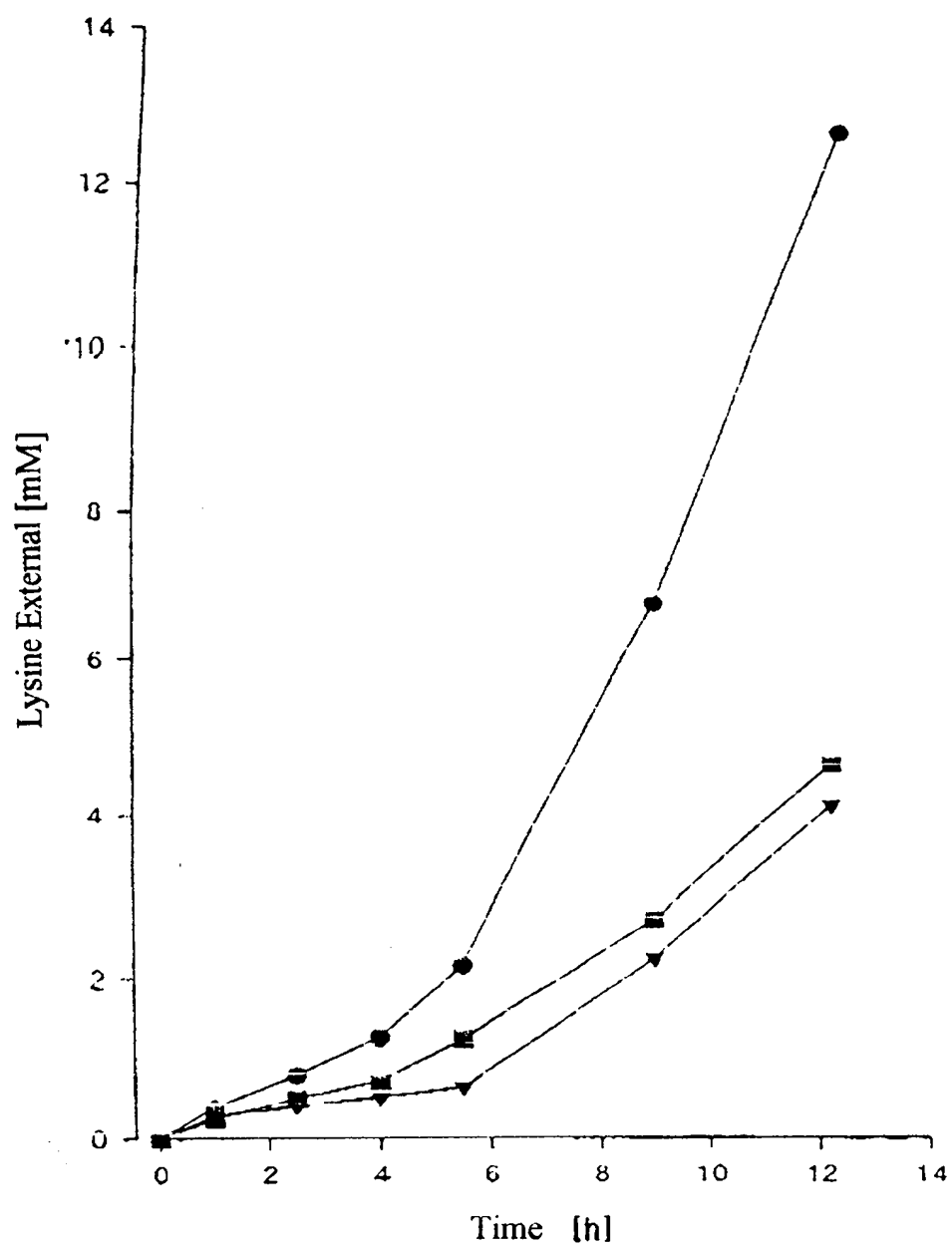


Figure 4

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RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR THE MICROBIAL PRODUCTION OF AMINO ACIDS BY BOOSTED ACTIVITY OF EXPORT CARRIERS

the specification of which is attached hereto or was filed on date _____ under SN: _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(a) and 35 U.S.C. 102 both as set forth on the reverse side hereof. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date (1) before that of the application on which priority is claimed or (2) if no priority claimed, before the filing date of this application.

PRIOR FOREIGN APPLICATION(S)

<u>Number</u>	<u>Country</u>	<u>Day/Month/Year Filed</u>	<u>Priority Claimed</u>
195 48 222 0	Germany	22/12/95	Yes

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior applications and the national or PCT international filing date of this application.

PRIOR U.S. OR PCT APPLICATION(S)

<u>Application Serial No.</u>	<u>Day/Month/Year filed</u>	<u>Status</u>
PCT/DE98/02485	18/12/96	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint

Klaus J. Bach (Reg.-No. 26,832), 4407 Twin Oaks Lane, Murrysville, PA 15668, Tel. 724-327-0684

to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent

1) Inventor's Signature _____

Inventor's Name: Marina Vrljic

Date 8.6.98
Citizenship: German

Residence (city): 52428 Jülich

State/Foreign Country: Germany

Post Office Address: Steinstrasser Allee 60, 52428 Jülich, Germany

2) Inventor's Signature _____

Inventor's Name: Lothar Eggeing

Date 8.6.98
Citizenship: German

Residence (city): 52428 Jülich

State/Foreign Country: Germany

Post Office Address: Eisenkamp 5, 52428 Jülich, Germany

3) Inventor's Signature _____

Inventor's Name: Hermann Sahm

Date 8.6.98
Citizenship: German

Residence (city): 52428 Jülich

State/Foreign Country: Germany

Post Office Address: Wendelinusstrasse 71, 52428 Jülich, Germany